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Immunohistopathology in the Guinea Pig Following Chronic Low-Level Exposure to Chemical Warfare Agents

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14. ABSTRACT
Guinea pigs exposed repeatedly to low levels of chemical warfare nerve agents exhibit behavioral changes, but no histopathological changes using traditional hematoxylin/eosin staining. To observe mild cytopathology, this study utilizes more sensitive methodologies of MAP-2 immunohistochemistry and Fluoro-Jade histofluorescence. Diet-unrestricted Male Hartley guinea pigs were exposed to 0.4 and 0.5 LD50 VX, soman, or sarin for 2 weeks, three weeks, or four weeks. A second group of diet-restricted guinea pigs was injected with 0.1, 0.2, or 0.4 LD50 VX for 1 week, 2 weeks, or 2 weeks followed by 1 week of recovery. Animals were euthanized with sodium pentobarbital and perfused with formalin. Brain sections were stained with MAP-2 and Fluoro-Jade. No changes in MAP-2 or Fluoro-Jade labeling were observed in diet-unrestricted animals. Diet-restricted animals exposed to 0.1 and 0.2 LD50 VX showed no alterations in MAP-2. Exposure to 0.4 LD50 VX resulted in increased MAP-2, however increased MAP-2 was not observed in 0.4 LD50 VX groups allowed to recover for 1 week. Results suggest that increased MAP-2 immunoreactivity could be due to an acute phase of increased neuronal activity as part of compensatory and repair mechanisms. Diet restriction may increase resistance of neurons to irreversible damage and facilitate recovery.

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MAP-2, Fluoro-Jade, diet restriction, low-dose, neurodegeneration

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Introduction

The organophosphorus (OP) chemical warfare nerve agents (CWNA) soman, sarin, and VX are highly toxic and lethal nerve gases. Impairment of learning and memory following OP poisoning was observed in humans (Sidell et al., 1974; Rosenstock et al., 1991). Animal studies have demonstrated that OP induces severe brain pathology in the piriform cortex, hippocampus, septum, entorhinal cortex, dentate gyrus, and amygdala (Britt et al., 2000; McDonough et al., 1987; Carpentier et al., 1991; McLeod, 1985). Deficits in cognition following OP toxicity are likely to be due to neuronal injury in the amygdala, hippocampus, and entorhinal cortex since these brain regions are involved in cognitive functions.

Behavioral studies showed that guinea pigs repetitively exposed to low-level CWNA exhibited behavioral changes (personal communication with Major Maurice Sipos). However, brain samples from these guinea pigs stained with classical hematoxylin and eosin (H&E) staining method did not exhibit any signs of histopathological changes. Using the H&E staining method, injured and dying cells appear red (or eosinophilic), indicating cytoplasmic protein coagulation. If proteins are not coalesced in degenerating cells, the H&E method is incapable of revealing cellular damage. Furthermore, it is possible that behavioral changes observed in CWNA-exposed guinea pigs could be due to structural changes (such as pruning of dendritic branches) that do not cause neuronal eosinophilia. Detecting these changes requires the use of more sensitive methodologies uniquely suited for observing mild cytopathology associated with neural deficits induced by low-level CWNA.

Microtubule-associated protein 2 (MAP-2) is the most abundant neuron-specific cytoskeletal protein in the brain, localized mostly in the dendritic processes (Caceres et al., 1984; De Camilli et al., 1984). Loss of MAP-2 immunoreactivity has been shown to be a sensitive marker for brain damage induced by soman toxicity (Ballough et al., 1995), cerebral ischemia (Kitagawa et al., 1989; Matesic and Lin, 1994) and traumatic brain injury (Folkerts et al., 1998; Posmantur et al., 1996). If repetitive exposures to low-dose CWNA cause neuronal injury, then brain sections from exposed animals should exhibit changes in MAP-2 immunoreactivity. In addition to examining MAP-2 changes, the presence of Fluoro-Jade (FJ) histofluorescence was evaluated. FJ is a fluorescent dye that specifically labels degenerating neurons (Schmued and Hopkins, 2000a,b; Schmued et al., 1997).

Materials and Methods

A total of 126 male guinea pigs (Crl:(HA)BR) were used in two separate studies. In the first study, 36 guinea pigs were placed on a food restricted-diet (Table 1). Animals were given 75% of the feeding recommendation (6 grams of food per 100 grams of body weight) to maintain body weight at approximately 400 grams. After 4 weeks of caloric restriction, animals were randomly assigned to treatment groups and were injected 5 days per week (Mon-Fri) with 0.1, 0.2, or 0.4 LD_{50} of the established LD_{50} dose of VX (9 μg/kg, s.c.) or saline (1 ml/kg) (Table 1). After 1 week of injections, 2 weeks of injections and 2 weeks of injections followed by 1 week of recovery, guinea pigs were deeply anesthetized by an overdose of sodium pentobarbital and perfused with 0.9% saline followed by 10% phosphate buffered formalin (PBF) (Fisher Scientific, Pittsburgh, PA).

Table 1. Assignment of Animals in the Diet-Restricted Study.

VX	1 wk injection	2 wk injection	2 wk injection/ 1 wk recovery	Total
Saline	3	3	3	9
0.1 LD50	3	3	3	9
0.2 LD50	3	3	3	9
0.4 LD50	3	3	3	9
Total	12	12	12	36

In the second study, ninety male guinea pigs were allowed to feed ad libitum. Seventy-two animals were randomly exposed to 0.4 and 0.5 LD_{50} of VX, soman (GD) or sarin (GB) for 2 weeks, 3 weeks, or 4 weeks (Table 2). The remaining 18 animals were used as control counterparts for each of the experimental combinations (Table 2). At the experimental end point, animals were euthanized and perfused as described above.

Table 2. Assignment of Animals in the Diet-Unrestricted Study.

VX, GD, or GB	2 wk injection	3 wk injection	4 wk injection	Total
Saline	6	6	6	18
0.4 LD50	4 X 3 agents	4 X 3 agents	4 X 3 agents	36
0.5 LD50	4 X 3 agents	4 X 3 agents	4 X 3 agents	36
Total	30	30	30	90

Brain Tissue Procurement

Following perfusion-fixation, brains were immediately removed from the skull and placed in 10% PBF at 4ºC for at least 18 hours, but no longer than 24 hours, to complete the fixation process. Care was exercised to avoid exerting any pressure on the skull during removal of the brains since pressure exerted on areas of insufficiently fixed brain tissue could induce neuronal injury (Cammermeyer, 1961). Brains were cut coronally into 3mm slabs from rostral to caudal using a guinea pig brain matrix. Individual brain slabs were placed in embedding cassettes and then processed in paraffin. Brain sections cut at 5µm were mounted on positively charged slides and allowed to air-dry at room temperature.

Microwave-Assisted MAP-2 Immunohistochemisrtry

Paraffin sections, deparaffinized in xylene, hydrated in graded ethyl alcohol and in distilled H_2O, were incubated in 5% H_2O_2 to suppress endogenous peroxidase activity. Following a 5 min rinse in running tap water and a 5 min rinse in distilled water, sections were pretreated in a microwave (Pelco 3440 Max, 800 watts, Ted Pella, Inc) for antigen retrieval (AR) (Kan et al., 2005; Pleva et al., 2002). Briefly, sections were boiled in the microwave in 10mM citric acid (pH 6.0) for 10

min (Sigma, St Louise, MO). Caution was taken to ensure that the brain sections did not dry during the microwave pretreatment procedure. Following pretreatment, sections were allowed to cool for 20 min at room temperature and then rinsed in PBS (Sigma, St Louise, MO).

Immunohistochemistry of MAP-2 (mouse monoclonal antibody, Clone AP18, 1:100; NeoMarkers, Fremont, CA) was performed using the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981). Following blocking in 5% horse serum for 30 min at 4°C, brain sections were incubated sequentially in primary antibody overnight (18 hrs) at 4°C, biotinylated secondary antibody (1:200; Vector Laboratory Inc., Burlingame, CA) for 1 hr at room temperature, and ABC solution (Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature. Immunoreaction product was developed with a solution containing diaminobenzidene and H_2O_2 (Sigma-Aldrich, St. Louis, MO) for 5 min. Brain sections were counterstained with either 0.8% cresyl violet acetate or Fluoro-jade B (FJ-B) for brain topography and morphology. Brain sections from guinea pigs exposed to 1.5 LD_{50} of soman, VX or sarin were used as positive control sections for changes in MAP-2 immunoreactivity and FJ labeling.

Results

Diet-Restricted Study

Brain sections of control animals showed typical patterns of MAP-2 immunoreactivity, mostly in dendritic processes and faintly in neuronal cell bodies (Figure1A). In experimental animals, no discernible alterations in the patterns of MAP-2 staining were observed in all brain regions after exposure to 0.1 and 0.2 LD_{50} VX for 1 week and 2 weeks. In contrast, animals exposed to 0.4 LD_{50} VX for 1 week and 2 weeks exhibited markedly increased immunoreactivity of MAP-2 in the CA2 subregion of the hippocampus (Figure 1B). Interestingly, this increased MAP-2 immunoreactivity was not observed in animals that received 0.4 LD_{50} VX for 2 weeks and then allowed to recover for 1 week. Negative control sections stained without MAP-2 primary antibody showed no MAP-2 immunostaining (Data not shown).

Diet-Unrestricted Study

During the course of this study, 17 guinea pigs unexpectedly died after receiving repeated doses of VX, GD and GB (Table 3). These animals exhibited no signs of nervous system disturbances, such as fasciculation, convulsions, or seizures. However, they appeared to be writhing soon after the injection. Necropsy revealed severe lower GI bleeding and intussusception (telescoping of one portion of the intestine into another) at the ileo-cecal junction.

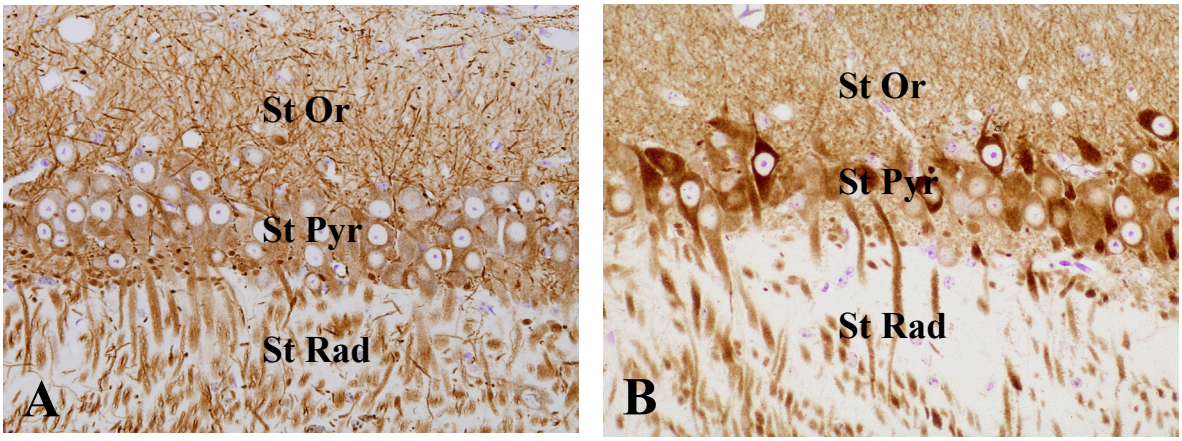
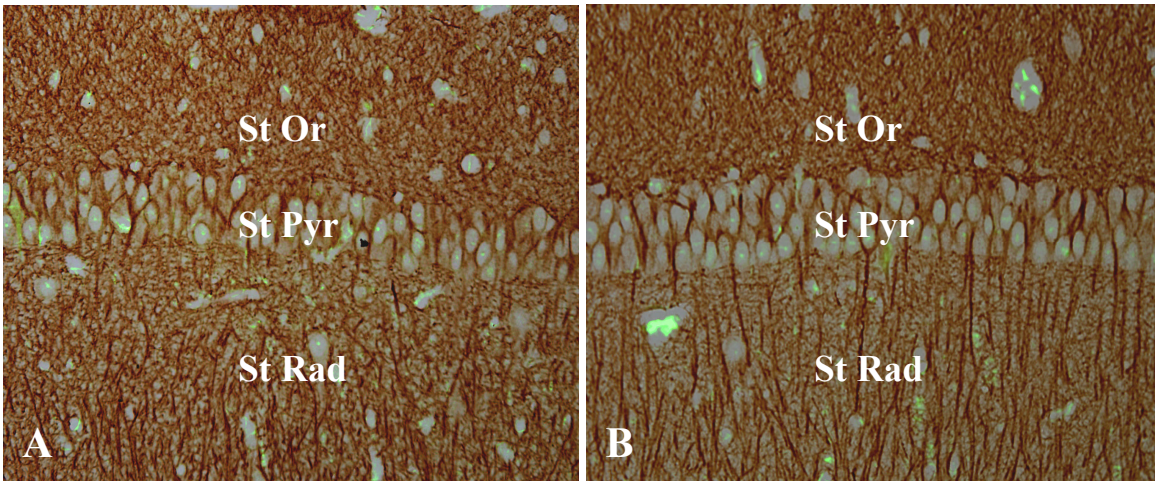


Figure 1. Immunoreactivity of MAP-2 in CA2 hippocampus of diet-restricted guinea pigs. (A) saline control, showing typical MAP-2 immunoreactivity, (B) 0.4 LD_{50} VX, showing increased MAP-2 immunoreactivity in pyramidal cell. St Or, Stratum Orien; St Pyr, Stratum Pyramidal; St Rad, Stratum Radiatum.

Table 3. Summary of Animals that Developed Intussusception After Repeated Exposures to Low-Dose Chemical Warfare Agents.

Agent	Dose	Number of Injections	Number of Animals
VX	0.4 LD_{50}	3	1 out of 9
VX	0.5 LD_{50}	2	1 out of 6
GB	0.4 LD_{50}	3	1 out of 12
GB	0.4 LD_{50}	6	2 out of 12
GB	0.4 LD_{50}	7	1 out of 12
GB	0.4 LD_{50}	9	1 out of 12
GB	0.5 LD_{50}	2	1 out of 13
GB	0.5 LD_{50}	5	1 out of 13
GB	0.5 LD_{50}	4	2 out of 13
GD	0.4 LD_{50}	5	2 out of 11
GD	0.4 LD_{50}	6	2 out of 11
GD	0.5 LD_{50}	4	1 out of 13
GD	0.5 LD_{50}	13	1 out of 13

Control sections showed MAP-2 immunoreactivity mostly in dendritic processes and no FJ-positive cells (Figure 2A). Similarly, no changes in MAP-2 immunoreactivity and FJ histofluorescence were observed in any brain regions of animals exposed to 0.4 LD50 and 0.5 LD_{50} VX, GD and GB for 2, 3 and 4 weeks (Figure 2B). Positive control sections from guinea pigs exposed to 1.5 LD_{50} VX exhibited loss of MAP-2 immunoreactivity and numerous of FJ-positive neurons (Figure 2C). It is important to point out that brain sections from animals that died from intussusception also did not show any alternations of MAP-2 immunoreactivity and FJ labeling.



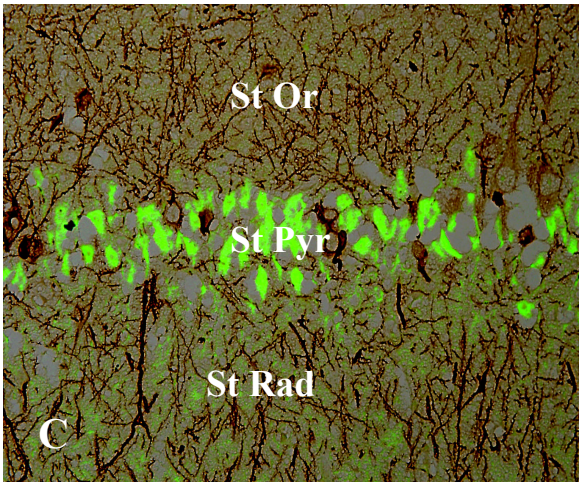


Figure 2. Representative sections of the hippocampus from freely fed guinea pigs stained with MAP-2 and FJ. (A) saline control sections, showing typical MAP-2 localization in dendritic branches and no FJ-positive neurons; (B) experimental sections from animals exposed to 0.5 LD_{50} VX for 4 weeks, showing no MAP-2 pathology and FJ-positive cells. Positive control sections (C) from animals exposed to 1.5 LD_{50} VX exhibited marked MAP-2 loss and many FJ-positive cells.

Discussion

The present study examined brain pathology induced by repeated exposures to low-dose chemical warfare nerve agents in the guinea pig. In the diet restricted study, animals exposed to 0.1 and 0.2 LD_{50} of VX did not show apparent changes in MAP-2 immunoreactivity. However, a robust increase in MAP-2 immunoreactivity in morphologically intact and altered neurons was observed in the CA2 hippocampus from animals exposed to 0.4 LD_{50} VX for 1 and 2 weeks. The increase in MAP-2 was not observed in animals that received 0.4 LD_{50} VX for 2 weeks and then allowed to recover for 1 week. MAP-2 has been suggested to play a key role in neuronal remodeling and plasticity after neuronal injury (Johnson and Jope, 1992). Taken together, the increased MAP-2 immunoreactivity could be due to an acute phase of increased neuronal activity as part of compensatory and repair mechanisms rather than irreversible brain injury.

In the diet-unrestricted study, no changes in MAP-2 immunoreactivity and FJ labeling were found in animals exposed to 0.4LD_{50} and 0.5 LD_{50} of VX, GB and GD for 2 weeks, 3 weeks and

4 weeks. These observations suggest that the doses of nerve agents and the duration of the experiment employed do not cause brain pathology. The results were different from the diet-restricted study, where increased MAP-2 immunoreactivity was found in the CA2 subregion of the hippocampus. The difference in the immunoreactivity of MAP-2 could be due to the difference in experimental conditions. In the diet-restricted study, guinea pigs were tested behaviorally for changes in acoustic startle response (ARS), whereas in the diet-unrestricted study, guinea pigs were not subjected to ARS testing. Whether or not the increase in MAP-2 immunoreactivity is related to diet-restriction or behavioral testing remains to be established.

Although no signs of neurotoxicity were detected, 24% of the exposed animals did not survive to complete the study. These animals were writhing soon after the last injection. Necropsy revealed severe gastrointestinal (GI) bleeding and intussusception at the ileo-cecal junction. We speculate that hyperactivity of the GI due to an increase in cholinergic transmission may contribute to the intussuception. Interestingly, intussusception is not observed in rats even after receiving convulsive doses of nerve agent (Kan et al., unpublished data). At the present, it is not clear why guinea pigs are prone to develop intussusception.

Conclusions

The present study revealed that 1) MAP-2 immunoreactivity is different between diet-restricted and freely fed animals after exposure to low-dose nerve agents, particularly VX and 2) guinea pigs are sensitive to developing intussusception. To gain better insights into the effects of exposure to chemical warfare nerve agents, additional research into the effects of diet restriction on changes in MAP-2 immunoreactivty and the susceptibility of guinea pigs to develop intussusception is warranted.

The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Anımal Care International. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the U.S. Army or the Department of Defense. In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (NRC 1996).

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